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Nitric oxide metabolites in patients with asthma: induced sputum versus blood

A.-S. JANG* AND I.-S. CHOI†

*Department of Internal Medicine, Seonam University College of Medicine and †Department of Internal Medicine, Chonnam University Medical School, Korea

Nitric oxide (NO) plays an important role in physiological regulation of the airways. The monitoring of airway inflammation has being observed in bronchial asthma directly, by sputum examination, and indirectly, by measurements in peripheral blood. To investigate the diagnostic value of these two methods, we compared NO metabolites in induced sputum and serum obtained in patients with asthma and control subjects. Hypertonic saline induced sputum and serum were obtained in 13 patients with asthma and 10 control subjects. NO metabolite level was assayed by using modified Griess reaction. Eosinophil cationic protein (ECP) was measured by fluoroimmunoassay, and detected interleukin (IL)-5 by a sandwich ELISA. The accuracy of the tests was measured by plotting the data in receiver operating characteristic (ROC) curves and comparing the area under the curve for NO metabolites. Asthmatic patients, compared with control subjects, had significantly higher NO metabolites in induced sputum ($1252{\cdot}5 \pm 203{\cdot}3$ mol l^{-1} *vs.* $557{\cdot}2 \pm 101{\cdot}5$ mol l^{-1}, $P<0{\cdot}01$) but not in serum. IL-5 in induced sputum was detected more frequently in patients with asthma than in control subjects [11/13 (84·6%) *vs.* 1/10 (10%), $P<0{\cdot}01$]. Asthmatic patients, compared with control subjects, had significantly higher ECP concentration in induced sputum ($1270{\cdot}0 \pm 197{\cdot}9$ g l vs. $154{\cdot}6 \pm 47{\cdot}4$ g l^{-1}, $P<0{\cdot}01$). There were significant positive correlations between NO metabolites in induced sputum and eosinophils, ECP in induced sputum ($r=0{\cdot}58$ $P<0{\cdot}05$; $r=0{\cdot}64$, $P<0{\cdot}01$) in patients with asthma but not in serum. The area under the ROC curve showed that NO metabolites in induced sputum (0·78) are more accurate marker than NO metabolites in serum (0·53) ($P<0{\cdot}05$). These findings suggest that NO metabolites in induced sputum is a more valuable indicator to monitor asthmatic airway inflammation than those in serum.

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Introduction

Nitric oxide (NO) plays an important role in physiological regulation of the airways and is produced in increased amounts in asthma (1). NO may amplify and perpetuate allergic inflammation by selective inhibition of helper T lymphocytes (Th1) which secrete interferon (IFN)-γ and in turn suppress the proliferation of Th2 lymphocytes (1,2). Eosinophilic inflammation in asthma is driven by Th2 lymphocytes which secrete interleukin (IL)-5 (1).

Previous studies have demonstrated the airway inflammation in bronchial asthma directly by bronchoscopic biopsies (3–5), bronchoalveolar lavages (6–7), or sputum examinations (8,9), and indirectly by measurements in peripheral blood (10,11).

A recent report (12) has shown that the concentration of NO derivatives is high in induced sputum in patients with asthma. Increased NO production in the airway has been reported in exhaled air of animals and asthmatic patients (13–15).

Serum concentrations of NO are elevated in patients with cirrhosis (16). This condition may result from excessive nitric oxide generated by the inducible enzyme. NO, which is produced by a variety of cells in the airways, probably plays a systemically physiological role in asthma. However, there are no studies examining whether NO production within the airways would be reflected systemically. Therefore the role of NO in blood in patients with asthma should be clarified. To the best of our knowledge, there has been no published data comparing NO metabolite level in induced sputum with those in serum. To investigate the diagnostic value of sputum and serum NO as marker of airway inflammation, we compared the level of NO metabolites in induced sputum and serum collected in asthmatic patients and control subjects.

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Correspondence should be addressed to Dr. An-Soo Jang, Division of Allergy and Pulmonology, Department of Internal Medicine, Seonam University College of Medicine, Seogu Marukdong 120-1, Kwangju, Korea, Zip code: 502-157. Fax: +82 62 382 3540; E-mail: jas877@chollian.net

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Patients and methods

STUDY SUBJECTS

Thirteen patients with asthma were recruited for this study from the Division of Allergy, Department of Internal Medicine, Chonnam University Hospital, Korea between March and December, 1997. The diagnoses of asthma were established in the patients by their symptoms of recurrent episodic wheezing, cough, and/or dyspnoea, accompanied either by methacholine airway hyper-responsiveness, with a baseline methacholine PC20$\leq$8 mg ml^{-1} (eight patients) or by a significant improvement in forced expiratory volume in 1 sec (FEV_1) (15%) following anti-asthma therapy (five patients). Seven patients were atopic. Eleven patients were treated with regular inhaled corticosteroids and β_2-agonists when needed, while two only used β_2-agonists when needed. No subject had respiratory infection for 4 weeks prior to the study. Ten control subjects, who were matched for age and sex, and volunteered for this study, had no history of any respiratory symptoms and had a $FEV_1 > 75\%$ predicted, a ratio of FEV_1 to forced vital capacity (FVC) $> 75\%$, and a normal methacholine airway responsiveness [provocation concentration of methacholine producing a 20% fall of FEV_1 (Pc20) > 16 mg]. The study was approved by Chonnam University Hospital Research Committee and all subjects signed the informed consent forms.

STUDY DESIGN

Airway inflammatory markers in hypertonic saline-induced sputum and serum were measured in patients with asthma and compared to those in control subjects. A questionnaire for symptoms and medications was given and a spirometry was also performed. Induced sputum and serum was collected, and skin prick tests and methacholine provocation tests were done. Clinical severity of asthma was classified by the method of the International Consensus Report (17). All measurements in sputum and serum were obtained by a person blind to the clinical characteristics of subjects.

Methods

PULMONARY FUNCTION TESTING

Spirometry was performed according to American Thoracic Society standards (18) using SensorMedics 2200 spirometer (Cardiopulmonary Care Company™, Yorba Linda, California, U.S.A.). The representative values for FVC and FEV_1 were selected according to International Thoracic Society criteria (19) and the reference values were taken from the reports by Choi *et al.* (20) and by Kim *et al.* (21).

NONSPECIFIC AIRWAY HYPER-RESPONSIVENESS

Methacholine challenge tests were carried out by the method described by Chai *et al.* (22) and the results were expressed as Pc20 [Pc20 FEV_1 was defined as a provoking dose of methacholine causing 20% fall in FEV_1 (forced expiratory volume in 1 sec)] in non-cumulative units.

ALLERGY SKIN TEST

Allergy skin prick tests were performed using 55 common allergen extracts. The results were read 15 min after the prick. The wheal and erythema size were measured, as maximum diameter and vertical length at the mid portion of maximum length, and presented as the mean value of the maximum diameter. Atopy is defined by one or more positive allergy skin prick tests.

SPUTUM INDUCTION AND PROCESSING

The sputum induction and examination were performed as described by Fahy *et al.* (23) and Popov *et al.* (24) with modification. All subjects were premedicated with two puffs of inhaled salbutamol (200 μg). Subjects inhaled 3% hypertonic saline solution aerosols generated by an ultrasonic nebulizer (NE-U03, OMRON Co., Tokyo, Japan) with maximum output of 0·15–0·3 ml min^{-1} and mass median aerodynamic diameter of 4·5 μm. Hypertonic saline was inhaled for 20–30 min according to the severity of asthma until an adequate volume of sputum expectorated. They were instructed to cough the sputum into a sterile plastic container. The volumes of samples and duration of sputum induction were recorded. FEV_1 was measured before, during and after induction of sputum. Sputum induction was stopped in each subject with a fall of the $FEV_1 > 15\%$.

Sputum was selected from saliva and processed within 2 h. Sputum was treated by adding equal volumes of 0·1% dithiothreitol (Sputalysin 10%; Gibco BRL, Grand Island, NY, U.S.A.) followed by equal volumes of Dulbecco's phosphate buffered saline (D-PBS). The sample was then mixed gently and placed in a shaking water bath at 37°C for 15 min to ensure complete homogenization. The sample was removed from the water bath periodically for further brief gentle mixing. The suspension was filtered through a gauze (1 mm pore size), the filtrate was centrifuged at 1500 rpm for 10 min and the supernatant was aspirated and stored in Eppendorf tubes at −70°C for later assay. The cell pellet was resuspended in D-PBS, 1000 μl and total non-squamous cells were counted in a modified Neubauer hemocytometer (AO, New York, U.S.A.). The cell suspension was adjusted to $0{\cdot}5 \times 10^5$ ml^{-1}, and then 50 μl of cell suspension was placed into cups of Sakura cytocentrifuge (Model CF-127, Tokyo, Japan), and two coded cytospins were prepared at 600 rpm at 5 min, air dried and stained by Diff-Quick® (Kookje Scientific Products, Tokyo, Japan) stain. Cell differentials of 400 non-squamous cells were performed by Diff-Quick stain on slides by two investigators who did not know the subject's history, and results were expressed as a percentage of the total non-squamous cell count.

BLOOD SAMPLING

Venous blood was collected in tubes containing 5·0 ml ethylenediaminetetraacetic acid (K3 Vacutainer BD, Rutherfold, NJ, U.S.A.) before sputum induction and differential white blood cell count was obtained with use of a Coulter STKS instrument (Coulter Corp., Hialeah, FL, U.S.A.). Serum was collected after blood coagulation for 1 h at room temperature. It was centrifuged at 20°C at 1500 rpm for 10 min and stored in Eppendorf tubes at −70°C for later assay.

NITRITE AND NITRATE ASSAY

Nitrite production was colorimetrically quantified after the Griess reaction as described by Greenberg *et al.* (25). One hundred μl of induced sputum supernatant and serum or standard were mixed with an equal volume of Griess reagent (1% sulfanilamide/0·1% naphthylethyllene dihydrochloride/2·5% phosphoric acid, Sigma Chemical Co., Sigma Chemical Co., St Louis, MO, U.S.A.) in duplicate microtitre wells at room temperature. Chromophore absorbance at 540 nm was determined. Nitrite concentration was calculated using sodium nitrite (Sigma Chemical Co., Sigma Chemical Co., St Louis, MO, U.S.A.) as a standard.

For assay of nitrate of samples, 200 μl of sputum supernatant and serum or standard containing 100 μl of 200 mM ammonium formate (including 100 mM HEPES, Sigma Chemical Co.) was reduced to nitrite at 37°C for 1 h by adding 100 l nitrate reductase [*E. coli* (ATCC25922), American Type Collection, Rockville, MD, U.S.A.], followed by centrifugation to precipitate non-reacting *E. coli* for 5 min, and then the nitrite was quantified as described above. The interassay and intraassay variablity are, respectively, ±4% and ±5% for NO metabolites.

IL-5 AND ECP MEASUREMENT

IL-5 was measured by quantitative sandwich enzyme immunoassay (Quantikine™; R&D Systems, Inc., Minneapolis, U.S.A.), as described by Dickason *et al.* (26). The concentration of ECP in 400 μl in the supernatant of induced sputum and serum was determined using fluoroimmunoassay (UniCAP system, Pharmacia AB, Uppsala, Sweden). Samples were analysed in duplicate. The limit of detection for IL-5 and ECP assays were 3 pg ml^{-1} and 2·0 μg l^{-1}, respectively.

STATISTICAL ANALYSIS

All data were analysed using the SPSS version 7·5 for Windows (Chicago, IL, U.S.A.). Data are expressed as mean ± SEM. Comparison of variables was performed using Student's *t*-test and the Mann–Whitney *U*-test. Pearson's correlations and Spearman's correlations were used to assess relationships between variables. Because the variables had a continuous scale of values, where the selection of a cutoff point was arbitrary, we determined the diagnostic accuracy of sputum and serum NO metabolites by generating a receiver operating characteristic (ROC) curve (27). The area under the curves (AUCs) were compared by the method of Hanley and McNeil (28). A *P*-value of $<0·05$ was considered significant.

Results

Subject characteristics are given in Table 1. All subjects tolerated the sputum induction procedure well. Asthmatic patients had significant lower FEV_1 and FEV_1/FVC than those of control subjects.

TABLE 1. Characteristics of patients with asthma

Patient No.	Age	Sex	Duration (yr)	Ig E (IU ml^{-1})	FEV_1 (% pred)	Pc20 M (mg ml^{-1})
1	24	F	15	266·9	78·8	NA
2	45	M	0·6	153·7	60·0	0·128
3	47	F	2	188·5	50·3	0·5801
4	39	M	5	NA	55·5	NA
5	38	F	1	30·1	58·0	0·9749
6	24	M	1	82·2	87·1	0·5600
7	26	M	5	30·1	76·2	0·1200
8	46	M	2	876·0	59·3	4·4820
9	54	F	3	4840·0	54·4	0·9279
10	40	M	1	1415·0	62·8	4·5140
11	59	M	1	1410·0	49·3	NA
12	51	F	10	260·0	64·5	NA
13	49	M	7	413·0	96·5	NA

Pc20 M:Pc20 methacholine [PC20 FEV_1 was defined as a provoking dose of methacholine causing 20% fall in FEV_1 (forced expiratory volume in one second)]; NA: no attributable.

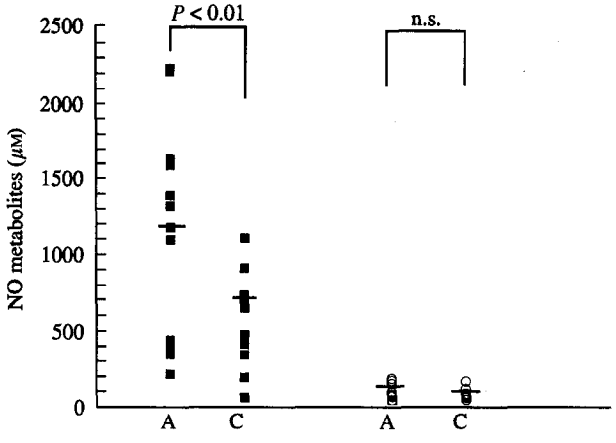

FIG. 1. Concentration of NO metabolites level in induced sputum and serum in asthmatic patients (A) and control subjects (C). Horizontal bars: mean values. ■: sputum; ○: serum.

INFLAMMATORY CELLS

The sputum contained less than 30% squamous epithelial cells, and had greater than 85% of cell viability. The proportion of eosinophils and neutrophils in induced sputum were significantly higher in patients with asthma than in control subjects (eosinophils: 48·6±5·4% *vs.* 1·8±0·2%, $P<0·01$; neutrophils: 18·5±2·4% *vs.* 9·4±1·9%, $P<0·01$), whereas the proportion of macrophages was lower (25·9±5·1% *vs.* 84·0±1·7%, $P<0·01$). The percentage of eosinophils is high due to patients selection including acute exacerbated patients. No significant differences were noted between asthmatic patients and control subjects in either total cell count or the absolute or relative numbers of lymphocytes and epithelial cells in induced sputum. The count of eosinophils in blood was significantly higher in patients with asthma than in control subjects (1069·0±440·5 μl^{-1} *vs.* 124·4±31·9 μl^{-1}, $P<0·01$).

NO METABOLITES, IL-5 AND ECP

The sputum from patients with asthma contained higher levels of NO metabolites (1252·5±203·3 μmol l^{-1} *vs.* 557·2±101·5 μmol l^{-1}, $P<0·01$; Fig. 1) than in serum. The AUC for each test revealed that the level of NO metabolites in induced sputum (0·78) was more sensitive and specific than the determination of serum NO metabolites (0·53) in differentiation of patients with asthma from control subjects (Fig. 2). IL-5 in induced sputum was detected more frequently in patients with asthma than in control subjects [11/13 (84·6%) *vs.* 1/10 (10%), $P<0·01$)]. Asthmatic patients had a significantly higher ECP concentration in induced sputum (1270·0±197·9 μg l *vs.* 154·6±47·4 μg l^{-1}, $P<0·01$) compared with control subjects.

RELATIONSHIP BETWEEN NO METABOLITES AND PHYSIOLOGICAL VARIABLES

NO metabolites in induced sputum were positively correlated with ECP in induced sputum ($r=0·64$, $P<0·01$; Fig. 3(a)). There was a significantly positive correlation between FEV_1/FVC and ECP in induced sputum ($r=0·59$, $P<0·01$). The level of NO metabolites in induced sputum tended to have positive correlation with FEV_1/FVC ($r=0·53$, $P=0·06$; Fig. 3(b)). However, there was no correlation between serum NO metabolites and lung function. No correlation was noted between inflammatory markers in induced sputum, serum and atopy score and airway hyper-responsiveness.

Discussion

The results of the current study show that NO metabolites in induced sputum compared with those in serum is a more useful, practical indicator in monitoring asthmatic airway inflammation.

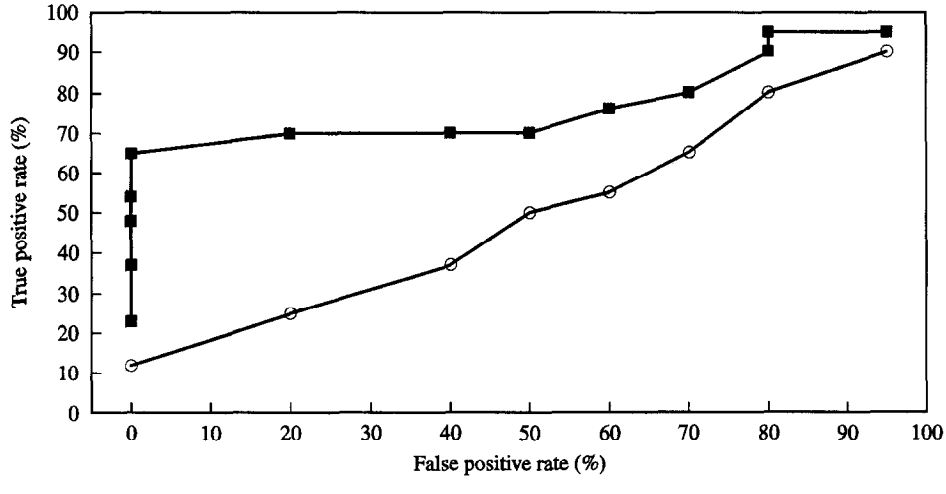

FIG. 2. NO metabolites in induced sputum and serum ROC curve. Plots that lie farthest to the 'northwest' represent more accurate values. -○-: serum, -■-: sputum.

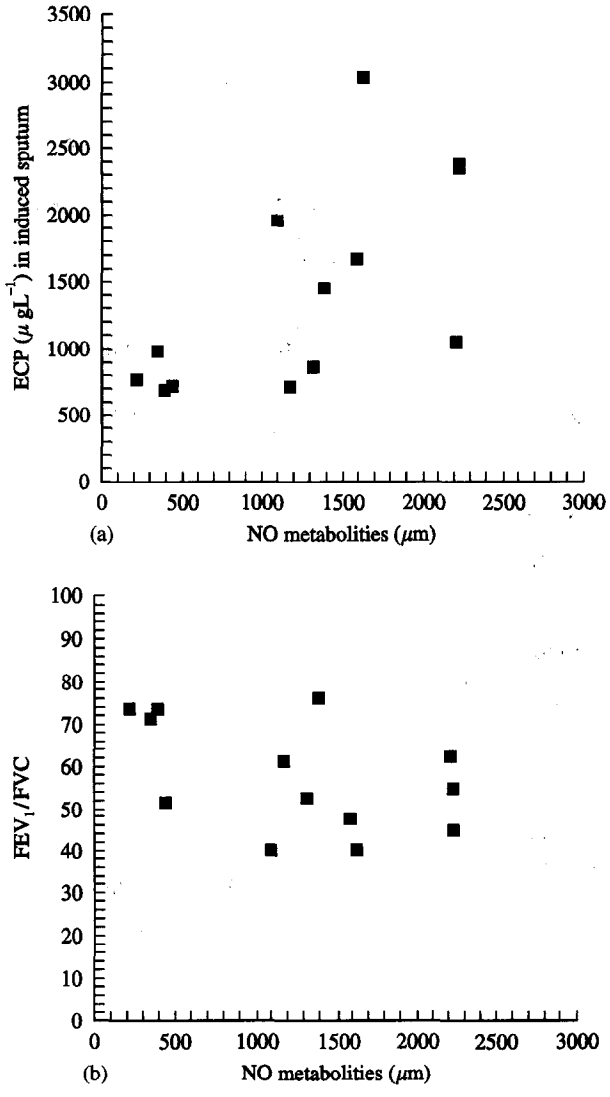

FIG. 3(a) Correlations between NO metabolites level in induced sputum and ECP in induced sputum in asthmatic patients (r=0·64; P=0·01). (b) Correlations between NO metabolites level in induced sputum and FEV_1/FVC in asthmatic patients (r=0·53; P=0·06).

Previous studies have demonstrated the airway inflammation in bronchial asthma directly by bronchoscopic biopsies (3–5), bronchoalveolar lavages (6–7) and sputum examinations (8,9), and indirectly by measurements in peripheral blood (10,11). This study is the first comparison of diagnostic accuracy between NO metabolites measured in induced sputum and peripheral blood samples. In the present study, higher levels of NO metabolites were shown in patients with asthma than in control subjects in induced sputum but not in serum. The AUC for each test revealed that the level of NO metabolites in induced sputum was more sensitive and specific than the determinations of serum NO metabolites in differentiation of patients with asthma from control subjects. These results imply that NO contributes to inflammation limited to tracheo-bronchial tree.

NO may amplify allergic inflammation by selective inhibition of T lymphocytes that secrete IFN-γ (Th1), which suppresses the proliferation of Th2 cells (28,34). In this study, although IL-5 not significantly correlated with NO metabolites, IL-5 significantly detected in the sputum of asthmatic subjects than in control subjects, suggesting that release of NO may contribute to allergic inflammation.

NO is endogenously derived from the amino acid *L*-arginine by three forms of the NO synthases (NOS). Two constitutive NOS are involved in physiological regulation of airway function, and inducible NOS is involved in inflammatory disease of the airways (1). Immunohistological studies have identified the presence of all three isoforms in human airways (29).

Several authors reported that NO in exhaled air increased in asthmatic subjects (30–31). High exhaled NO concentrations in asthmatic patients may reflect induction of NOS. Measurement of exhaled NO concentration may be clinically useful in detection and management of cytokine mediated inflammatory lung disorders (32).

Kanazawa *et al.* (12) reported that measurement of NO derivatives in induced sputum may be useful for assessing allergic inflammation in airways. NO levels in induced sputum were significantly correlated with percentages of shedding epithelial cells. NO generated by inducible NOS (iNOS) in airway epithelial cells combined avidly with superoxide anion generated by inflammatory cells in airways to form peroxynitrite, which may have inflammatory effects directly and through the generation of toxic hydroxyl radicals that may contribute to airway epithelial shedding in asthma (12). NO may indirectly exacerbate the airway inflammatory response by inducing shedding of epithelial cells (12), because damage to airway epithelial cells may occur even in patients with relatively mild asthma, thereby exposing afferent nerve endings (3). We (33) reported that the level of NO metabolites were increased in the tracheo-bronchial secretion of asthmatic subjects and was paralleled by severity of asthma. Measurement of NO metabolites in induced sputum may be used for monitoring the degree of airway inflammation in asthmatics. However, in contrast to a previous report (12), this study did not demonstrate a significant correlation between epithelial cells and NO metabolites. However, we showed that the proportion of eosinophils positively correlated with NO metabolites, suggesting a possibility that there is another source which releases NO metabolites. It has been reported that cells potentially capable of generating NO in the lungs include macrophages, neutrophils, mast cells, nonadrenergic inhibitory neurons, fibroblasts, vascular smooth muscle cells, pulmonary artery and venous endothelial cells and pulmonary epithelial cells (32–33). Further study will be needed to evaluate the role of NO metabolites in induced sputum in asthma.

Serum concentrations of NO are elevated in patients with cirrhosis (16). This condition may result from excessive nitric oxide generated by the inducible enzyme. We evaluated whether NO had a probability of systemically playing a physiological role in asthma. In this study we didn't find that NO levels in serum were increased. These

results imply that NO has an effect on inflammation limited to the respiratory tract in asthma.

In accordance with a previous report (12), we found that NO metabolites in induced sputum tended to correlate with airway obstruction (FEV_1/FVC) but not in serum. Therefore induced sputum NO metabolites could serve as a useful marker for monitoring disease activity in asthmatic patients. We did not find a correlation between NO metabolites in induced sputum and serum, and non-specific airway hyper-responsiveness. The significance of increased NO metabolites in induced sputum needs to be evaluated in such a population with correlations to symptoms and treatment.

We conclude that NO metabolites in induced sputum have a more valuable diagnostic value than those in serum in monitoring airway inflammation in asthma.

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